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Bacterial protein degradation by different rumen protozoal groups[1]

A. Belanche, G. de la Fuente, J. M. Moorby, and C. J. Newbold[2]

Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, United Kingdom SY23 3DA

ABSTRACT: Bacterial predation by protozoa has the most deleterious effect on the efficiency of N use within the rumen, but differences in activity among protozoal groups are not completely understood. Two in vitro experiments were conducted to identify the protozoal groups more closely related with rumen N metabolism. Rumen protozoa were harvested from cattle and 7 protozoal fractions were generated immediately after sampling by filtration through different nylon meshes at 39ºC, under a CO_2 atmosphere to maintain their activity. Protozoa were incubated with ^{14}C-labeled bacteria to determine their bacterial breakdown capacity, according to the amount of acid-soluble radioactivity released. *Epidinium* tended to codistribute with *Isotricha* and *Entodinium* with *Dasytricha*; therefore, their activity was calculated together. This study demonstrated that big Diplodiniinae had the greatest activity per cell (100 ng bacterial CP per protozoa and hour), followed by *Epidinium* plus *Isotricha* (36.4), small Diplodiniinae (34.2), and *Entodinium* plus *Dasytricha* (14.8), respectively. However, the activity per unit of protozoal volume seemed to vary, depending on the protozoal taxonomy. Small Diplodiniinae had the greatest activity per volume (325 ng bacterial CP per protozoal mm^3 and hour), followed by big Diplodiniinae (154), *Entodinium* plus *Dasytricha* (104), and *Entodinium* plus *Dasytricha* (25.6). A second experiment was conducted using rumen fluid from holotrich-monofaunated sheep. This showed that holotrich protozoa had a limited bacterial breakdown capacity per cell (*Isotricha* 9.44 and *Dasytricha* 5.81 ng bacterial CP per protozoa and hour) and per protozoal volume (5.97 and 76.9 ng bacterial CP per protozoal mm^3 and hour, respectively). Therefore, our findings indicated that a typical protozoal population (10^6 total protozoa/mL composed by *Entodinium* sp. 88%, *Epidinium* sp. 7%, and other species 4%) is able to break down ~17% of available rumen bacteria every hour. *Entodinium* sp. is responsible for most of this bacterial breakdown (70 to 75%), followed by *Epidinium* sp. (16 to 24%), big Diplodiniinae (4 to 6%), and small Diplodiniinae (2 to 6%), whereas holotrich protozoa have a negligible activity (*Dasytricha* sp. 0.6 to 1.2% and *Isotricha* sp. 0.2 to 0.5%). This in vitro information must be carefully interpreted, but it can be used to indicate which protozoal groups should be suppressed to improve microbial protein synthesis in vivo.

Key words: bacterial breakdown, nitrogen metabolism, protein degradation, rumen protozoa

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INTRODUCTION

Microbial protein synthesized in the rumen represents the main AA source for ruminants, even though up to 50% of this microbial protein is degraded to ammonia as a result of N turnover in the rumen (Koenig et al., 2000). Thus, reducing ruminal N turnover represents a strategy to improve microbial yield and N use by ruminants.

Rumen protozoa represent an appreciable proportion of the rumen biomass (38%, Leng and Nolan, 1984) and have much longer generation time than bacteria (8 to 36 h vs. about 20 min). To maintain their numbers, rumen protozoa have different survival strategies, based on the maintenance of low passage rates. Entodiniomorphids remain attached to fibrous material, whereas holotrich can rapidly migrate from the reticulum to the rumen (Abe et al., 1981). As consequence of such sequestration, up to 74% of protozoa are lysed within the rumen and bacterial predation by rumen protozoa is considered to have a major detrimental effect on rumen N metabolism (Ffoulkes and Leng, 1988).

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[2]Corresponding author: cjn@aber.ac.uk
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Defaunation and suppression of protozoal numbers (by using saponins, tannins, or certain lipids) are strategies to reduce internal microbial protein degradation to improve net availability to the animal (Eugène et al., 2004). Total defaunation can, however, limit certain beneficial effects of rumen protozoa (i.e., fiber degradation and VFA production). Bacterial breakdown seems to vary among protozoal groups; thus, there is a pressing need to identify those groups most important in rumen N metabolism to develop strategies to suppress target protozoal groups.

Rumen protozoa are classified by 2 major groups; entodiniomorphids and holotrich. Thus, 2 in vitro trials were conducted to investigate their activity. The aims of this study were to 1) develop a method to generate different protozoal populations without compromising their activity, and 2) quantify bacterial breakdown capacity of the most common protozoal groups per cell and per protozoal volume.

MATERIAL AND METHODS

Animals were managed by trained personnel and protocols were approved by the authority of the UK Animals Act, 1986. Both experiments described below were conducted in duplicate (2 batches in consecutive weeks) to generate a more robust data set.

Exp. 1: Study of Total Fauna Activity

Preparation of Labeled Bacteria. The activity of protozoal populations was measured in vitro from the breakdown of ^{14}C-labeled rumen bacteria, as described by Wallace and McPherson (1987). Total rumen bacteria were isolated from rumen fluid obtained from 4 barren rumen-cannulated Holstein-Frisian cows fed at maintenance level (10.5 kg DMI composed of 0.67:0.33 ratio perennial ryegrass hay and ground barley, on a DM basis). Rumen contents were sampled before the morning feeding, pooled, and filtered through a double layer of gauze and stored anaerobically at 39ºC. Protozoa and plant material were sedimented by centrifugation at 1,000 × *g* for 10 min at 39°C and bacteria were collected from the supernatant. Rumen bacteria were cultured for 3 d on medium no. 2 of Hobson (1969) at 39ºC by daily transfer of culture into fresh media. During the 2 d before the experiment, bacterial culture was isotope-labeled by growing in the same medium containing ^{14}C-leucine (1.44 μ Ci/8 mL tube). On the day of the experiment, labeled cultures were centrifuged (3,000 × *g* for 15 min) and the supernatant discarded. Sedimented bacteria were washed once in fresh unlabeled medium and centrifugation was repeated. Then, sediment was resuspended in 8 mL of simplex-type salts solution (**STS;** Williams and Coleman, 1992), containing ^{12}C-leucine (5 mmol/L) to prevent reincorporation of released ^{14}C-leucine by bacteria. Bacterial resuspension was subsampled to determine its radioactivity and N content (Lowry et al., 1951). Finally, 0.5mL of this bacterial resuspension was inoculated in each protozoal tube as fed.

Protozoal Fractionation and Counting. Rumen protozoa were also isolated from samples obtained as described above. Rumen fluid was sampled for protozoal counting. The remaining rumen fluid was transferred into the lab within 30 min after extraction and was always kept at 39ºC under anaerobic conditions. Rumen fluid was diluted (1:2 vol/vol) with STS buffer (Williams and Coleman, 1992) containing 2 g/L of glucose, distributed in sedimentation funnels, and incubated for 1 h to allow protozoal cells to settle. The sediment was collected and filtered through a 250-μm pore diameter nylon mesh to remove plant material and fractionated consecutively through 6 nylon meshes (80-, 60-, 45-, 35-, 20-, and 5-μm pore diameters, Sefar AG, Hinterbissaustrasse, Switzerland), which generated 6 protozoal fractions (F80, F60, F45, F35, F20, and F5, respectively). Fractionation was conducted at 39ºC and under CO_2 gas, and rumen protozoa were filtered gently without using a vacuum pump to minimize cell damage and maintain protozoal activity. Fractions were washed thoroughly and diluted with 50 ml of STS buffer. An additional protozoal fraction (**Fmix**) was generated by pooling equal volumes (7 mL) of all 6 fractions. All protozoal fractions were sampled (in triplicate) to determine N concentration (Lowry et al., 1951) and conduct a protozoal counting [1 mL sample + 9 mL formalin 9.25% (vol/vol) and NaCl 9% (wt/vol)]. All protozoal species were identified and quantified by optical microscope, using the procedure described by Dehority (1993) and modified by de la Fuente et al. (2006). Length and width of every protozoal species were determined as an average of at least 10 individuals and protozoal volume was estimated considering a cylindrical protozoa shape. All protozoal species were classified in 6 major groups, according to their morphology and phylogenetic origin (Table 1): *Isotricha* sp., *Dasytricha* sp., *Entodinium* sp., *Epidinium* sp., small Diplodiniinae, and big Diplodiniinae. This latter group contained only 2 species (*Eudiplodinium maggii* and *Metadinium medium*).

Rate of Bacterial Protein Degradation. Protozoal fractions (8 mL) were incubated in quadruplicate with ^{14}C-labeled bacteria (0.5 mL) in Hungate tubes. Incubation was carried out at 39ºC and tubes were sampled every hour (0, 1, 2, 3, and 4 h), using a syringe with a 23-gauge needle. Samples (0.5 mL) were acidified (by adding 125 μL of trichloroacetic acid at 25% wt/vol) and centrifuged (11,000 × *g* for 5 min at 39°C). Supernatant (200 μL) was diluted with 2 mL of scintillation fluid to determine the radioactivity released by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). A negative control (^{14}C-labeled bacteria without protozoa) was also incubated to estimate

Table 1. Mean size (±SD) and protozoal species distribution (% of total protozoal cells) in the different protozoal fractions isolated from normally faunated cows (Exp. 1) or holotrich-monofaunated sheep (Exp. 2)

Item	Dimensions, µm		Fmix	Protozoal fractions[1]					
	Length	Width		F80	F60	F45	F35	F20	F5
Exp. 1 concentration, × 10^5 protozoa/mL			3.8 ± 0.6	0.8 ± 0.5	2.0 ± 1.0	2.5 ± 0.8	2.0 ± 0.9	11 ± 2	3.2 ± 0.7
Entodinium									
E. nanellum	23 ± 2	12 ± 1	1.43	-	-	-	0.05	0.63	2.78
E exiguum	25 ± 3	16 ± 1	0.92	-	-	-	-	0.61	2.58
E. rostratum	35 ± 4	20 ± 1	0.63	-	-	-	0.03	0.51	2.11
E. parvum	33 ± 2	22 ± 1	1.26	0.10	0.12	0.30	0.34	1.24	3.13
E. bicornutum	31 ± 4	25 ± 2	0.46	-	-	-	-	0.13	1.07
E. caudatum f. caudatum	35 ± 7	28 ± 8	0.54	-	-	-	0.03	0.46	0.98
E. simplex	44 ± 4	25 ± 3	0.49	-	-	-	-	0.32	0.95
E. dubardi	40 ± 11	27 ± 6	1.53	0.24	0.12	0.13	0.45	1.07	6.29
Entodinium pisciculum	38 ± 7	29 ± 2	0.28	-	-	-	-	0.14	1.22
E. caudatum f. lobosospinosum	38 ± 7	29 ± 8	0.13	-	-	-	-	0.16	0.77
E. rectangulatum f. caudatum	35 ± 8	31 ± 5	0.64	-	-	-	-	0.52	1.20
Entodinium bicarinatum	40 ± 4	30 ± 2	1.78	-	-	-	0.03	2.21	4.87
E. caudatum f. dubardi	41 ± 14	30 ± 12	1.40	-	-	-	0.08	1.14	5.78
E. longinucleatum	54 ± 7	33 ± 2	0.71	-	-	-	-	1.02	1.90
E. anteronucleatum f. dilobum	67 ± 10	45 ± 4	0.35	-	-	-	-	0.12	0.57
Epidinium									
E. quadricaudatum	93 ± 13	45 ± 6	2.91	7.60	7.32	11.44	5.95	0.48	-
E. ecaudatum	127 ± 3	43 ± 2	0.19	0.35	1.01	0.21	0.14	0.02	-
E. cattanei	99 ± 14	51 ± 8	0.78	1.53	1.13	0.92	0.56	0.03	-
E. parvicaudatum	121 ± 26	46 ± 5	5.03	5.80	6.72	12.9	11.0	1.27	-
E. caudatum	126 ± 19	46 ± 6	2.65	5.12	4.87	7.63	4.25	0.51	-
E. tricaudatum	119 ± 14	49 ± 19	0.37	1.98	4.22	3.41	0.56	0.03	-
E. hamatum	127 ± 15	52 ± 6	0.27	-	0.12	0.10	0.07	0.02	-
Small Diplodiniinae									
Diplodinium monolobosum	58 ± 6	39 ± 6	0.75	-	0.17	0.09	0.19	0.41	0.55
Eudiplodinium rostratum	60 ± 10	43 ± 7	0.57	-	-	-	0.08	0.35	1.07
Eudiplodinium bovis	63 ± 5	44 ± 3	2.45	1.21	0.64	1.32	2.14	4.81	1.43
Ostracodinium trivesiculatum	66 ± 6	45 ± 4	5.83	0.14	0.48	2.64	4.34	12.0	2.79
Ostracodinium gracile	66 ± 5	48 ± 6	1.28	0.65	0.60	0.26	1.45	2.49	1.03
Ostracodinium mammosum	75 ± 15	46 ± 8	-	-	-	-	-	0.22	-
Diplodinium anacanthum	80 ± 7	50 ± 7	0.09	0.85	0.60	0.62	0.54	0.25	0.05
Eudiplodinium neglectum monolobum	71 ± 16	56 ± 7	1.29	-	0.05	1.37	1.82	1.04	0.17
Ostracodinium rugoloricatum	105 ± 14	48 ± 7	1.12	-	-	0.31	0.96	1.73	0.32
Diplodinium dentatum	74 ± 7	57 ± 4	0.37	0.40	0.60	0.84	0.75	0.38	-
Ostracodinium venustum	96 ± 13	51 ± 6	0.71	-	0.24	0.22	0.64	0.91	-
Enoploplastron triloricatum	86 ± 18	54 ± 11	0.14	-	0.20	-	0.32	0.16	-
Diplodinium tetracanthum	78 ± 4	57 ± 3	1.12	1.41	2.50	5.06	1.74	0.25	-
Eudiplodinium dilobum	85 ± 11	55 ± 10	2.22	0.40	0.17	1.15	3.67	1.89	-
Metadinium minorum	82 ± 12	60 ± 11	0.34	1.25	0.74	0.75	0.51	0.43	0.24
Diplodinium triacanthum	78 ± 8	62 ± 6	1.20	1.45	2.11	3.08	1.42	0.22	0.05
Diplodinium anisacanthum	82 ± 9	66 ± 8	2.43	1.41	2.49	7.93	8.84	1.70	0.12
Diplodinium diacanthum	82 ± 8	66 ± 8	1.13	1.61	2.89	2.82	1.26	0.38	-
Ostracodinium clipeolum	110 ± 12	58 ± 5	0.27	-	-	0.09	0.32	0.33	-
Metadinium affine	105 ± 13	59 ± 14	0.09	0.29	0.20	-	-	-	-
Diplodinium monacanthum	85 ± 10	66 ± 7	1.13	0.65	1.01	1.27	1.69	0.05	0.02
Ostracodinium obtusum	133 ± 10	68 ± 8	0.89	-	0.36	0.35	0.43	1.01	-
Elytroplastron bubali	132 ± 17	77 ± 10	-	-	-	-	0.03	-	-

Table 1. Continued.

Item	Dimensions, µm		Fmix	Protozoal fractions					
	Length	Width		F80	F60	F45	F35	F20	F5
Big Diplodiniinae									
Eudiplodinium maggii	132 ± 17	88 ± 7	5.30	30.3	24.1	3.79	0.16	0.05	0.07
Metadinium medium	169 ± 7	115 ± 7	0.75	9.85	0.85	-	-	-	-
Isotricha									
I. prostoma	189 ± 21	77 ± 21	8.90	17.3	21.4	18.5	18.7	2.71	0.56
I. intestinalis	170 ± 13	84 ± 16	1.02	4.90	9.15	5.84	3.11	-	-
Dasytricha ruminantium	80 ± 9	35 ± 3	33.8	3.24	2.88	4.72	21.4	53.6	55.3
Exp. 2 concentration, × 10^5 protozoa/mL			2.2 ± 0.2		0.3 ± 0.2	0.8 ± 0.3		6.9 ± 0.8	1.2 ± 0.0
Isotricha									
I. prostoma	190 ± 27	84 ± 1	1.91		9.43	19.4		0.68	0.56
I. intestinalis	195 ± 21	105 ± 21	12.1		89.9	76.9		0.26	-
Dasytricha ruminantium	72 ± 6	37 ± 4	86.0		0.63	3.66		99.1	99.4

[1]Protozoal fractions (F80, F60, F45, F35, F20, and F5) were generated by a sequential filtration through nylon meshes with a pore size of 80, 60, 45 35, 20, and 5 µm diam., respectively. Fmis was generated by pooling equal volumes of the previous fractions. Each value is the mean of 8 incubation tubes.

the rate of bacterial auto-degradation. Bacterial breakdown at each incubation time was expressed as the percentage of acid-soluble radioactivity released relative to the total radioactivity present in the initial labeled bacteria. The percentage of bacterial degradation rate per hour in each incubation tube was calculated by simple linear regression and protein degradation rate was determined by multiplying these figures by the CP content in the bacterial substrate. Incubation tubes with great protozoal concentrations and/or with the presence of very active species consumed most of the labeled bacteria after a few hours of incubation, giving nonlinear responses at later incubation times; therefore, only the linear part was considered under such conditions. The protozoal activity of each incubation tube was expressed based on protozoal concentration (10^6 cells) or volume (mm^3) to make incubation tubes with different protozoal numbers comparable. The effect of the protozoal fraction (fix effect; F80, F60, F45, F35, F20, F5, and Fmix) on the bacterial CP degradation was analyzed by ANOVA, blocking by batch (1 and 2), and considering each incubation tube as an experimental unit (random effect; 4 replicates per protozoal fraction). Mean separation was conducted using Fisher's protected LSD test, with statistical significance declared at $P < 0.05$.

Bacterial CP breakdown by each protozoal group [*Entodinium* sp. (**EN**) vs. *Epidinium* sp. (**EP**) vs. small Diplodiniinae (**SmD**) vs. big Diplodiniinae (**BD**) vs. *Isotricha* sp. (**IS**) vs. *Dasytricha* sp. (**DA**)] was estimated by multiple linear regression as follows:

$$B_i = \mu_i + EN_i \cdot B_{EN} + EP_i \cdot B_{EP} + SmD_i \cdot B_{SmD} + BD_i \cdot B_{BD} + IS_i \cdot B_{IS} + DA_i \cdot B_{DA} + e_i$$

where B_i (response variable) is the bacterial CP breakdown per protozoal cell (or volume) and hour observed in each protozoal fraction (i = F80, F60, F45, F35, F20, F5, or Fmix); µ is the bacterial breakdown in absence of protozoa; EN_i, EP_i, SmD_i, BD_i, IS_i, and DA_i (fitted terms) are the observed proportions of every protozoal type; B_{EN}, B_{EP}, B_{SmD}, B_{BD}, B_{IS}, and B_{DA} are the regression coefficients that determine the bacterial protein breakdown capacity of each protozoal group; and e is the residual error of the model. The best-fit values in the multiple linear regressions were calculated by using the least square approach in the statistical software GenStat (VSN International Ltd, Hemel Hempstead, Herts, UK). The proportion of variance accounted for the model and residual SmD was used to assess the performance of the equation. Estimated activities of the different protozoal groups were compared using a *t*-test, as described by Sokal and Rohlf (1995). Finally, the amount of bacterial protein degraded in the rumen by the different protozoal groups was calculated by multiplying the estimated activity of each protozoal group by their respective abundance in the rumen of the experimental cows considering.

Exp. 2: Study of Holotrich Protozoa Activity

A second experiment was conducted to investigate the effect of rumen holotrich protozoa on bacterial protein breakdown, following the protocol described above. Sheep were used as protozoal donors instead of cattle because generation of monofaunated cattle was not feasible in our facilities. Eight lambs were isolated from their mothers within 24 h after lambing and maintained protozoa free by avoiding protozoal transmission from adult ruminants. When lambs became adults, they were orally inoculated with holotrich pure cultures (*I. prostoma*, *I. intestinalis*, and *D. ruminantium*) to generate the holotrich-monofaunated sheep used as donors. Sheep received a standard mixed diet fed at maintenance (1.6 kg DMI composed of a 0.67:0.33 ratio perennial ryegrass hay and ground barley, on a DM basis). Rumen contents (200 mL per sheep) were withdrawn by orogastric intubation before the morning feed-

ing. The protozoa fractionation procedure was the same as described above and 6 protozoal fractions were generated (F80, F60, F45, F35, F20, and F5), but only 4 of them were used. Fractions F60 and F45 were chosen to be representative of *Isotricha* sp. and fractions F20 and F5 were chosen to be representative of *Dasytricha* sp. Subsamples of these 4 fractions were pooled to generate a mixed protozoal population (Fmix). Incubation procedure and data analysis were the same as Exp. 1.

RESULTS

Exp. 1: Study of Total Protozoa Activity

Protozoal Fractionation. Rumen fluid from experimental cows contained a type B protozoal population made up of 50 different protozoal species varied in size and volume (from 2,337 to 1,745,079 μm^3/cell). Mean ruminal concentration of protozoa were $1.8 \times 10^6 \pm 0.9 \times 10^6$ cells/mL; *Entodinium* sp. was the most abundant protozoa (87.8 ± 2.8% of the total population), followed by *Epidinium* sp. (6.7 ± 2.2%), *Dasytricha* (2.4 ± 1.3%), small Diplodiniinae (2.0 ± 1.1%), *Isotricha* (0.6 ± 0.5%), and big Diplodiniinae (0.5 ± 0.4%). The protozoal fractionation procedure was successful and filtration through progressive smaller pore sizes resulted in a subsequent decrease in average protozoal size (Table 1). Furthermore, no protozoal cells were detected in the last filtrate (<5µm diameter). It was observed that CP content per protozoon was positively correlated (R^2 = 0.78) with protozoal size, whereas fractions F80 and F5 contained protozoa with the greatest CP content per volume ($P < 0.001$, Table 2).

It was considered that protozoal activity should be similar for protozoal species that belong to the same subfamily and have similar size. Protozoal species were therefore classified in 6 major groups to simplify data interpretation (mean volume in 1000 μm^3/cell); 15 species belonged to *Entodinium* sp. (21 ± 2.8), 7 to *Epidinium* sp. (189 ± 5.4), 23 to small Diplodiniinae (188 ± 42.0), 2 to big Diplodiniinae (878 ± 117.5), 2 to *Isotricha* sp. (895 ± 4.3), and 1 to *Dasytricha* sp. (76 ± 1.0). The differences in cell size among protozoal groups resulted in a dissimilar distribution pattern among the protozoal fractions (Figure 1). Big Diplodiniinae (mainly appeared in F80 and F60) and small Diplodiniinae (F45, F35, and F20) had very particular distribution patterns. However, this fractionation procedure was not specific enough to make a clear separation between *Epidinium* sp. and *Isotricha* sp. (both associated with F60, F45, and F35), as well as for *Entodinium sp.* and *Dasytricha sp.* (F20 and F5).

Measurement of Bacterial Breakdown. The amount of bacteria degraded by protozoa increased linearly (R^2 > 0.99) over the incubation time considered (4 h) in all experimental units. The rate of bacterial degradation in absence of protozoa was negligible (<0.06% of the initial radioactivity was released each hour), but it ranged 1.3 to 36%/h in the presence of protozoa. Similar effects of the protozoal fraction on the bacterial breakdown rate were found when rate was expressed either as proportion of radioactivity released or bacterial CP degraded (Table 2), because similar CP contents were observed in the labeled bacteria in both batches (101 ± 7.1 µg bacterial CP/mL). The greatest bacterial breakdown rate per protozoal cell was found in F80, whereas F5 showed the least ($P <$ 0.001). In terms of bacterial breakdown per unit of protozoal volume, protozoa present in fractions F80, F45, F35,

Table 2. Average protozoal size, CP content, and bacterial breakdown rate in several protozoal fractions isolated from normally faunated cows (Exp. 1)

Protozoal fractions[1]	Fmix	F80	F60	F45	F35	F20	F5	SEM	*P*-value
Size, 10^3 μm^3/protozoa	241^{e}	718^{a}	565^{b}	388^{c}	333^{d}	119^{f}	65.2^{g}	11.6	<0.001
Protozoal CP content									
ng/protozoa	31.9^{c}	111^{a}	57.1^{b}	32.7^{c}	29.2^{c}	9.40^{d}	10.2^{d}	5.00	<0.001
µg/protozoa mm^3	130^{bc}	170^{a}	101^{d}	86^{d}	95^{d}	82^{d}	158^{ab}	11.6	<0.001
Bacterial breakdown rate[2]									
%/(10^6 protozoa·h)	19.6^{c}	95.4^{a}	45.0^{b}	42.1^{b}	42.9^{b}	17.5^{c}	4.89^{d}	3.32	<0.001
%/(protozoal mm^3·h)	0.08^{b}	0.13^{a}	0.08^{b}	0.12^{a}	0.12^{a}	0.15^{a}	0.07^{b}	0.014	0.001
Bacterial CP breakdown rate[3]									
ng/(protozoa·h)	19.8^{c}	96.5^{a}	45.5^{b}	42.6^{b}	43.4^{b}	17.7^{c}	0.49^{d}	3.36	< 0.001
ng/(protozoal mm^3·h)	79.9^{b}	134^{a}	78.1^{b}	122^{a}	126^{a}	148^{a}	71.8^{b}	14.54	0.001
ng/(100 ng protozoal CP·h)	6.10^{b}	8.39^{b}	8.16^{b}	14.7^{a}	14.0^{a}	18.5^{a}	4.67^{b}	1.773	< 0.001

$^{a–g}$Within a row, means without a common superscript differ ($P < 0.05$).

[1]Protozoal fractions (F80, F60, F45, F35, F20, and F5) were generated by a sequential filtration through nylon meshes with a pore size of 80, 60, 45 35, 20, and 5 µm diam., respectively. Fmix was generated by pooling equal volumes of the previous fractions. Each value is the mean of 8 incubation tubes.

[2]Expressed as % of initial bacterial radioactivity released per hour.

[3]Expressed as amount of initial bacterial CP degraded per hour.

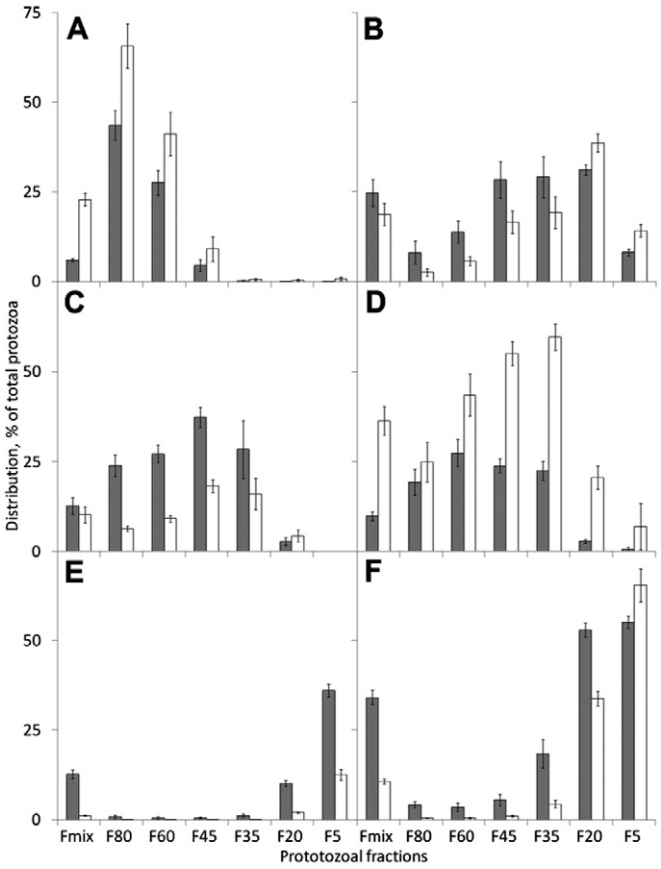

Figure 1. Proportions of A) big Diplodiniinae, B) small Diplodiniinae, C) *Epidinium* sp., D) *Isotricha* sp., E) *Entodinium* sp., and F) *Dasytricha* sp. in different protozoal fractions generated by sequential filtration through nylon meshes with a pore size of 80, 60, 45 35, 20, and 5 μm diam. Values and SD (error bars) are expressed as percentages of total protozoal cells (gray columns) and total protozoal volume (white columns). Each value is the average of 8 incubation tubes.

and F20 showed greater activity than those in F60 and F5 ($P = 0.001$). Similar results were observed in terms of bacterial CP released per unit of protozoal CP. Midsize fractions (F45, F35, and F20) showed twice the activity observed for larger-size protozoa fractions (F80 and F60) and more than 3 times that observed for F5.

Due to the similar distribution pattern among certain protozoal groups during the fractionation procedure, the bacterial breakdown capacity of those protozoal groups with similar distribution was estimated as a common group (i.e., *Epidinium* sp. plus *Isotricha* sp. or *Entodinium* sp. plus *Dasytricha* sp; Table 3). By doing this, it was observed that big Diplodiniinae sp. had the greatest bacterial breakdown capacity per cell, being 3 times more active than small Diplodiniinae sp. or *Epidinium* sp. plus *Isotricha* sp., and being 6.7 times more active than *Entodinium* sp. plus *Dasytricha* sp ($P = 0.026$). When bacterial breakdown was expressed per unit of protozoal volume, small Diplodiniinae had the greatest activity and *Epidinium* sp. plus *Isotricha* sp. had the lowest ($P = 0.032$).

Exp. 2: Study of Holotrich Protozoa Activity

Protozoal fractionation procedure was also successful in Exp. 2 and a clear separation by size was observed (Table 4). Average cell size was 18.6 times greater in protozoa present in fractions F60 and F45, than in F20 and F5 ($P < 0.001$). In agreement with Exp. 1, big protozoa (F60 and F45) showed a greater CP content per cell than small protozoa (F20 and F20), whereas the opposite was true in terms of protozoal volume ($P < 0.001$). The optical examination of protozoal fractions showed that *Isotricha* sp. comprised practically the entire protozoal population in fractions F60 and F45, whereas *Dasytricha* sp. was the most important group in F20 and F5 (Table 1).

A linear release of the acid-soluble radioactive label was observed during the first 4 h of incubation (average $R^2 > 0.98$). Bacterial breakdown rate ranged between 0.43% and 10.2%/h, and negligible degradation was observed in the negative control (<0.05% of the initial radioactivity was released each hour). A greater bacterial breakdown per protozoal cell was observed in F60 and F45, than in F20 and F5 ($P < 0.001$), but the opposite was true in terms of protozoal volume (Table 4). The results were similar, regardless

Table 3. Estimation of the bacterial breakdown capacity of several protozoal groups (Exp. 1)

Item[1]	Big Diplodiniinae	Small Diplodiniinae	*Epidinium* plus *Isotricha*	*Entodinium* plus *Dasytricha*	Eq. performance[2]		*P*-value
					Adj. r^2	rsd	
Bacterial breakdown rate[3]							
%/(10^6 protozoa·h)	$99.2^{a} \pm 35.9$	$33.8^{bc} \pm 12.4$	$36.0^{ab} \pm 12.2$	$14.7^{c} \pm 5.5$	70.1	4.62	0.026
%/(protozoal mm^3·h)	$0.15^{b} \pm 0.04$	$0.32^{a} \pm 0.08$	$0.03^{c} \pm 0.03$	$0.10^{b} \pm 0.08$	73.8	4.32	0.032
Bacterial CP breakdown rate[4]							
ng/(protozoa·h)	$100^{a} \pm 36.3$	$34.2^{bc} \pm 12.5$	$36.4^{ab} \pm 12.4$	$14.8^{c} \pm 5.6$	70.1	4.67	0.026
ng/(protozoal mm^3·h)	$154^{b} \pm 44.3$	$325^{a} \pm 83.1$	$25.6^{c} \pm 28.5$	$104^{b} \pm 80.6$	73.8	4.37	0.032

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

[1]Protozoal activity in each protozoal group (mean ± SD) was estimated by multiple linear regression, based on total protozoal activity (response variable) and proportion of each protozoal group in each protozoal fraction (explanatory variable). Estimations were performed using 8 incubation tubes from each 7 protozoal fractions ($n = 56$).

[2]Equation performance is explained by the adjusted r^2 (variance accounted) and residual SD (rsd).

[3]Expressed as % of initial bacterial radioactivity released per hour.

[4]Expressed as amount of initial bacterial CP degraded per hour.

Table 4. Average protozoal size and CP content in protozoal fractions isolated from holotrich monofaunated sheep (Exp. 2)

Protozoal fractions[1]	Fmix	F60	F45	F20	F5	SEM	*P*-value
Size, 10^3 µm^3/ protozoa	289^{c}	1617^{a}	1504^{b}	86^{d}	81^{d}	15.7	<0.001
Protozoal CP content							
ng/protozoa	25.6^{bc}	111^{a}	54.7^{b}	11.9^{c}	32.5^{bc}	10.44	<0.001
µm/protozoa	96.3^{bc}	69.2^{cd}	36.8^{d}	138^{b}	407^{a}	14.78	<0.001
mm^3							
Bacterial breakdown rate[2]							
%/(10^6 protozoa·h)	16.5^{bc}	33.1^{a}	18.5^{b}	13.6^{c}	10.9^{c}	1.31	<0.001
%/h per protozoal mm^3	6.57^{b}	2.05^{c}	1.24^{c}	15.7^{a}	13.5^{a}	1.103	<0.001
Bacterial CP breakdown rate[3]							
ng/(protozoa·h)	6.64^{bc}	13.6^{a}	7.74^{b}	5.87^{c}	4.20^{d}	5.67	<0.001
ng/(protozoal mm^3·h)	22.8^{b}	7.14^{c}	4.31^{c}	54.6^{a}	47.1^{a}	3.84	<0.001
ng/(100 ng protozoal CP·h)	2.62^{b}	1.83^{c}	1.56^{d}	5.33^{a}	1.35^{e}	0.004	<0.001

$^{a–d}$Within a row, means without a common superscript differ ($P < 0.05$).

[1]Protozoal fractions (F80, F60, F45, F35, F20, and F5) were generated by a sequential filtration through nylon meshes with a pore size of 80, 60, 45 35, 20, and 5 µm diam., respectively. Fmix was generated by pooling equal volumes of the previous fractions. Each value is the mean of 8 incubation tubes.

[2]Expressed as % of initial bacterial radioactivity released per hour.

[3]Expressed as amount of initial bacterial CP degraded per hour.

Table 5. Estimation of bacterial breakdown capacity of *Isotrichia* sp. and *Daystrichia* sp· (Exp. 2)

			Eq. performance[2]		
Item[1]	*Isotricha* sp.	*Dasytricha* sp.	Adjusted r^2	rsd	*P*-value
Bacterial breakdown rate[3]					
%/(10^6 protozoa·h)	19.8^{a} ± 2.94	13.7^{b} ± 0.40	94.4	0.779	0.050
%/(protozoal mm^3·h)	0.01^{b} ± 0.002	0.18^{a} ± 0.005	94.4	0.776	<0.001
Bacterial CP breakdown rate[4]					
ng/(protozoa·h)	9.44 ± 2.02	5.81 ± 0.27	86.9	0.536	0.09
%/(protozoal mm^3·h)	5.97^{b} ± 1.26	76.9^{a} ± 3.48	87.0	0.534	<0.001

^{a,b}Within a row, means without a common superscript differ ($P < 0.05$).

[1]Protozoal activity in each protozoal group (mean ± SD) was estimated by multiple linear regression, based on total protozoal activity (response variable) and proportion of each protozoal group in each protozoal fraction (explanatory variable). Estimations were performed using 8 incubation tubes from each 5 protozoal fractions (n = 40).

[2]Equation performance is explained by the adjusted r^2 (variance accounted) and residual SD (rsd).

[3]Expressed as % of initial bacterial radioactivity released per hour.

[4]Expressed as the amount of initial bacterial CP degraded per hour.

of whether bacterial breakdown was calculated either as radioactivity released or , bacterial CP degraded.

This successful fractionation made it feasible to estimate *Isotricha* sp. and *Dasytricha* sp. activities with a greater accuracy than observed in Exp. 1 (Table 5). *Isotricha* sp. had a slightly greater bacterial breakdown capacity per cell compared with *Dasytricha* sp. (1.45 times greater in terms of percentage of radioactivity released, $P = 0.050$; and 1.62 times greater in terms of bacterial CP degraded, $P = 0.09$). In contrast, *Dasytricha* sp. had a much greater activity per protozoal volume than observed in *Isotricha* sp (between 13 and 14 times, $P < 0.001$).

The rate of bacterial breakdown by protozoa was comparable in both experiments. Therefore, it was hypothesized that holotrich activity estimated in sheep could be used to resolve the overlap of protozoal species observed in cattle. Making this assumption and subtracting the bacterial CP degraded as a consequence of holotrich activity, the activities of *Entodinium* sp. and *Epidinium* sp. in Exp. 1 were recalculated. This new estimation, which must be carefully interpreted, showed a similar bacterial breakdown per *Entodinium* sp. cell but with a greater variability (14.5 ± 14.6 µg bacterial CP/10^6 protozoa and hour). However, *Epidinium* sp. activity was determined with better accuracy and the value increased substantially with respect to that initially calculated in common with *Isotricha* sp. (58.4 ± 21.0 µg bacterial CP/10^6 protozoa and hour).

DISCUSSION

Exp. 1: Methodological Aspects

The protocol used to generate different protozoal populations according to their size was successful; however, an understanding of its potential limitations is vital to interpret our results. During the isolation procedure, glucose was added to encourage polysaccharide formation by protozoa and facilitate a rapid sedimentation. Holotrich protozoa have, however, a greater capacity to engulf and store polysaccharides than other protozoal groups, leading to a greater recovery (Heald et al., 1952). This led to a greater holotrich proportion in our protozoal fractions than is generally observed in the rumen. The opposite was true for *Entodinium* as consequence of their slow sedimentation (Williams and Coleman, 1992). Secondly, rumen protozoa are flexible and can squeeze through pores smaller than their apparent cell size. This ability is especially pronounced in protozoa without skeletal plates and led holotrich protozoa to appear in fractions where the average size of entodiniomorphids was about 4 times greater. Thirdly, Sylvester et al. (2005), using a similar sedimentation-filtration protocol to that used here, observed minimal contamination of protozoal samples with rumen bacteria. However, bacterial contamination in protozoal isolates has been described when small pore sizes are used (Belanche et al., 2011b).

This may explain the greater N content observed in fraction F5 and could result in a slight underestimation of the protozoal activity in this particular fraction.

The authors are aware that isolation of specific protozoal groups from a mixed ciliate population is feasible by using more sophisticated procedures, such as density gradient centrifugation, sedimentation through buffered gradients, or migration to electric field (Williams and Coleman, 1992). Incubations with mannose (Lockwood et al., 1988) or wide spectrum antibiotics (Heald et al., 1952) have also been described as effective procedures to lyse holotrich protozoa or protozoal-associated bacteria, respectively. Nevertheless, in preliminary studies (data not shown), we observed that the time required and cell damage caused by these procedures can compromise protozoal viability and activity; therefore, they were disallowed. Our findings show that an improved version of the protocol described by Williams and Coleman (1992) did not allow complete isolation of particular protozoal groups but did allow isolation of active protozoal populations composed of different proportions of protozoal groups.

Rumen protozoa can obtain N from plant material by assimilation of ammonia into AA (Newbold et al., 2005) and indeed engulfment of other protozoa; however, the greater availability of rumen bacteria seems to make this the preferred N source for protozoa. After digestion, a substantial proportion of this bacterial N is released into the medium (mainly as AA and ammonia) and has been used as an indicator of bacterial breakdown (Wallace and McPherson, 1987; Newbold et al., 1997). All protozoal species engulf mixed rumen bacteria and those grown in vitro engulf them slightly faster (1.5 to 17.6 times) than those grown in vivo (Williams and Coleman, 1992). Several factors seem to determine the rate of bacterial uptake by protozoa, being dependent of the protozoa considered (i.e., species, starvation, and adaptation to in vitro conditions), bacterial inoculum (i.e., density, adhesion to substrates, or bacterial morphology), and medium used (pH and concentration of salt, AA, or other nutrients; Coleman and Sandford, 1979; Wallace and McPherson, 1987). In the present paper, these sources of variation were minimized by incubating protozoa with liquid-associated bacteria, considered more likely to be predated than solid-associated bacteria (Gutierrez and Hungate, 1957; Orpin and Letcher, 1984). In vitro labeling of inoculum may modify the bacterial community but allows rumen protozoa to develop a similar behavior to that found in vivo, because a mixed bacterial community was available and a nonselective predation has been described (de la Fuente et al., 2011). Finally, antagonism between certain protozoal species and changes in their feeding behaviors have been described when new species are inoculated in the rumen (Dehority, 2003). Under our experimental conditions, rumen protozoa were isolated from a stable protozoal community. Moreover, the same bacterial density was generated in all tubes and incubation was carried out in a buffer without other nutrients to enhance protozoal predation and minimize possible changes in feeding behaviors.

Bacterial Breakdown by Particular Protozoal Groups

Among the diverse protozoal species that inhabit the rumen, *Entodinium* sp. are by far the most abundant (Dehority, 2003), representing 80 to 98% of the total population (33 to 37% of volume). This high abundance compensates for the low *Entodinium* activity per cell. Thus, a positive correlation has been described between the concentration of *Entodinium* sp. and rate of bacterial degradation in vitro (Wallace and McPherson, 1987), whereas it was found to be negatively related to microbial N flow in vivo (Ivan et al., 2000b ; Ivan, 2009), suggesting that *Entodinium* sp. is the most important protozoan in terms of absolute bacterial CP breakdown. In agreement with this, our results (Figure 2) suggest with conventional livestock diets 70 to 75% of the bacterial CP degraded by the protozoa in the rumen may be due to the *Entodinium sp.* and *Dasytricha sp.* activity, with the former genus being responsible for most of this degradation. In Exp. 1, the combined estimation of bacterial breakdown by *Entodinium* sp. plus *Dasytricha* sp. had a lower CV when expressed per protozoal cell (37%) than per protozoal volume (77%). This suggests that *Entodinium sp* and *Dasytricha sp.* have similar activities per cell but not per volume, because the former group is 3.6 times smaller than the latter. Similarly, *Epidinium* sp. and *Isotricha* sp. seem to have a similar activity per cell but not per volume, because the former group is 4.7 times smaller than the latter.

Although with slight differences, cattle and sheep share most protozoal species (Dehority, 2003). Therefore, considering a similar activity in holotrich from sheep and cattle, we re-estimated the activity of *Epidinium* and *Entodinium* without interference of holotrich protozoa. This new determination did not substantially modify the activity of *Entodinium* sp. *Epidinium* sp. activity was, however, calculated with a better accuracy than previously estimated. *Epidinium* sp. are defined as cellulolytic protozoa (Ivan et al., 2000a), but they also have substantial capacity to engulf and digest bacterial pure cultures (Coleman and Sandford, 1979), and to produce extracellular lysozyme-like enzymes, which contribute, to some extent, to bacterial breakdown (Coleman and Laurie, 1974). Our estimations suggest that *Epidinium* sp. (representing about 7% of total protozoa) is the second most important protozoal group, being responsible for 16 to 24% of the bacterial CP degraded by protozoa in the rumen (Dehority, 2003).

Eudiplodinium maggii and *Metadinium medium* are also cellulolytic protozoa and their presence leads to increases in the fiber digestibility in the rumen (Ivan et al., 2000a). As was expected, big Diplodiniinae showed the greatest bacte-

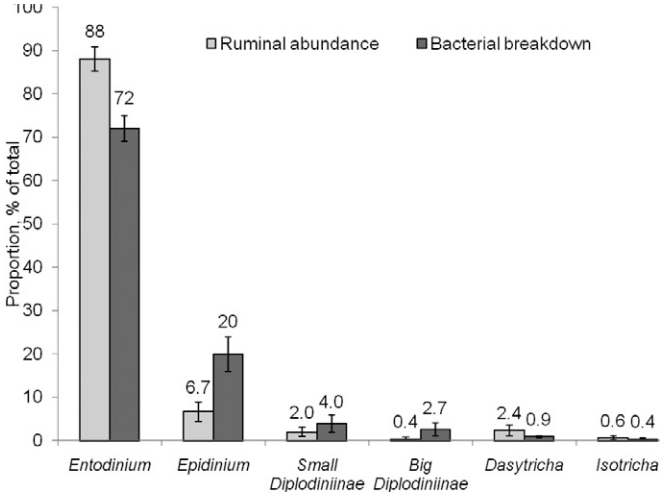

Figure 2. The relative abundance of the 6 major protozoal groups in rumen of cattle (Exp. 1) and bacterial breakdown attributed to each of these protozoa groups.

rial breakdown capacity per cell (6.8 times greater than *Entodinium*). In spite of their great activity, their concentration in the rumen rarely exceeds 1% of total protozoa (Dehority, 2003). Therefore, not more than 6% of the bacterial CP degraded in the rumen can be attributed to the big Diplodiniinae in ruminants fed typical diets. This limited activity of big Diplodiniinae in rumen N recycling may explain the lack of differences in terms of rumen ammonia concentration and microbial protein synthesis when *Eudiplodinium maggii* colonized the rumen (Ivan et al., 2000a).

The different sizes of the 23 protozoal species classified as small Diplodiniinae led them to be present in most of the protozoal fractions. Surprisingly, the activity of this fraction appeared relatively constant with a low SE, suggesting a similar activity for all species included in this group. In particular, small Diplodiniinae had similar rates of bacterial breakdown activity per cell to that observed in *Epidinium* sp. plus *Isotricha* sp. group, but the former group had the greatest activity per protozoal volume. Small Diplodiniinae often represent from 1 to 3% of total protozoal cells in the rumen (Dehority, 2003) and our data suggest that they are responsible for 2 to 6% of bacterial CP degraded by protozoa.

Finally, we found substantial differences in the CP content of different protozoal populations. This could lead to substantial errors in estimates of protozoal N flow to the intestine if a constant CP content is assumed for the entire protozoal population (Belanche et al., 2011a).

Exp. 2: Bacterial Breakdown by Holotrich Protozoa

Because it was not possible to determine the bacterial breakdown capacity of holotrich protozoa in Exp. 1 due to our inability to physically separate holotriches from other protozoal groups, Exp. 2 was conducted using holotrich-monofaunated sheep. It is known that holotrich protozoa have a chemotaxis to polysaccharides and are able to engulf and store these sugars for subsequent use (Williams and Coleman, 1992). Holotrich produce H_2, establish metabolic interactions with methanogens (Vogels et al., 1980; Tokura et al., 1997), and are able to modify the rumen fermentation pattern (Jouany et al., 1995), but little is known about their bacterial breakdown capacity (Gutierrez and Hungate, 1957). *Isotricha sp.* cells from sheep had 19.3 times greater volume than *Dasytricha sp.*; however, the former species showed a bacterial breakdown activity per cell only slightly greater than the latter (about 1.5 times greater). Therefore, *Dasytricha sp.* was substantially more active than *Isotricha sp.* per unit of volume (14 times greater). The *Isotricha sp*. group comprised 2 species (*I. intestinalis* and *I. prostoma*), but *Dasytricha sp*. comprised just 1 species, leading to a better estimation of its activity. In typical rumen fermentation, *Isotricha* sp. represent 0.3 to 1% of the total protozoa population, whereas *Dasytricha sp.* is present in greater concentrations (2 to 4%). Thus, our data suggest that 0.2 to 0.6% and 0.6 to 1.2% of the bacterial CP degraded by protozoa in the rumen is due to *Isotricha sp.* and *Dasytricha sp.*, respectively. These data agree with previous literature (Ivan et al., 2000b ; Ivan, 2009), where a limited effect of presence of holotrich on the microbial N flow and rumen N metabolism was observed. Their preferential use of soluble protein and free AA as N sources, instead of rumen bacteria, may explain this finding (Wallis and Coleman, 1967; Onodera and Kandatsu, 1970).

In an early in vitro study, Coleman and Sandford (1979) estimated that 6 to 16.2% of rumen bacteria are engulfed and digested by the rumen protozoal population every hour at a protozoal concentration of 2×10^6 cells/mL; this represents 10 g of bacterial N per day in sheep fed restricted high grain rations. Slightly smaller values were observed by Leng and Nolan (1984) in vivo, indicating that the turnover of N between bacteria and protozoa ranged between 3.7 and 6 g/d in sheep fed different diets. Our measurements indicate that in our experimental cows, between 14 and 34 g of bacterial N may be broken down each day by the action of rumen protozoa (based on 175 L of rumen content). This might suggest a slight underestimation of protozoal activity in our in vitro experiments compared with in vivo results (Leng and Nolan, 1984). Several factors, such as differences in rumen protozoal numbers, high bacterial availability in vivo, and difficulty in simulating the rumen environment in vitro, could explain these differences (Williams and Coleman, 1992).

Conclusions

This study described a fractionation procedure that allows generating active protozoal populations to investigate their activity in vitro. Our findings indicated that the amount of bacterial degraded per protozoal cell depends

on the protozoal size. Big protozoa (i.e., big Diplodiniinae) had the greatest activity, followed by *Epidinium* plus *Isotricha* and small Diplodiniinae, whereas small protozoa (i.e., *Entodinium* plus *Dasytricha)* had the least activity per cell. The activity per protozoal volume seems, however, to vary, depending on the protozoal group considered, with Diplodiniinae and Holotriches having the greatest and least activity, respectively. This information might suggest which protozoal groups should be targeted to improve the efficiency of N utilization by ruminants. However, more research is required to validate these results in vivo and to investigate the activity of other protozoal fauna types.

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